Probing the Role of the Trivalent Metal in Phosphate Ester Hydrolysis: Preparation and Characterization of Purple Acid Phosphatases Containing Al^{III}Zn^{II} and In^{III}Zn^{II} Active Sites, Including the First Example of an Active Aluminum Enzyme

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Abstract: Purple acid phosphatases contain a dinuclear $Fe^{3+}M^{2+}$ center in their active site ($M = Fe^{2+}$ or Zn^{2+}). To resolve the specific role of the ferric ion in catalysis, a series of metal-substituted forms of bovine spleen purple acid phosphatase (BSPAP) of general formula $M^{III}Zn^{II}$-BSPAP has been prepared, in which the trivalent metal ion was systematically varied ($M^{III} = $ Al, Fe, Ga, and In). The activity of the AlZn-BSPAP form was only slightly lower ($k_{cat} \approx 2000$ s^{-1}) than that of the previously reported GaZn and FeZn forms ($k_{cat} \approx 3000$ s^{-1}). The InZn form was inactive. The kinetics parameters and pH profile of AlZn-BSPAP were remarkably similar to those of FeZn-BSPAP and GaZn-BSPAP, but AlZn-BSPAP was readily distinguished from the GaZn and FeZn forms by its 50−70-fold lower inhibition constant for fluoride. These results are not, at first sight, consistent with intrinsic properties of the trivalent metal ions as they are known from coordination chemistry. In particular, aluminum has generally been believed to be of little use as a Lewis acid in the active site of an enzyme because of the slow ligand exchange rates typically observed for aluminum complexes. The present results are thus in conflict with this general wisdom. The conflict can be resolved either by assuming that the protein modulates the properties of the aluminum ion such that ligand exchange rates are substantially enhanced and thus not rate-limiting, or by assuming a catalytic mechanism in which ligand exchange does not take place at the trivalent metal ion.

Purple acid phosphatases (PAPs) are unique enzymes in that they use a dinuclear $Fe^{III}Fe^{II}$ or $Fe^{III}Zn^{II}$ center to catalyze the hydrolysis of phosphate esters.[1−3] They are further characterized by their purple color, which is due to a tyrosinate-to-Fe^{3+} charge-transfer band, their acidic pH optimum, and their insensitivity to tartrate, which is an inhibitor of other classes of acid phosphatases. PAPs have been isolated from mammalian, plant, and microbial sources.[1] The mammalian enzymes, of which uteroferrin (Uf) and bovine spleen purple acid phosphatase (BSPAP) are the best-studied examples, contain two antiferromagnetically coupled iron ions. The plant purple acid phosphatase from kidney beans (KBPAP) contains one iron and one zinc. Despite this difference in metal content, the mammalian and plant PAPs display similar enzymatic and spectroscopic properties. One of the irons in BSPAP and Uf can be replaced by zinc without drastic effects on the phosphatase activity,[4−9] while the zinc ion in KBPAP can be replaced by

iron, again without major effects on the catalytic activity.[10,11] The kidney bean purple acid phosphatase is the only PAP whose X-ray structure has been reported.[12,13] The PAPs belong to a much larger group of enzymes that catalyze phosphate ester hydrolysis and share a sequence motif incorporating most of the metal ligating amino acids in KBPAP.[13−16] X-ray structures of two Ser/Thr-specific protein phosphatases, protein phosphatase 1[17,18] and protein phosphatase 2B (calcineurin),[19,20]

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which also have this sequence motif, show the presence of a dinuclear metal center which resembles that of KBPAP, although the identity of the metals is still the subject of some debate.[21−23]

The metal centers in Uf, BSPAP, and KBPAP have been studied extensively by various spectroscopic techniques, including EPR,[5,7,9,10,24,25] Mössbauer,[25−30] resonance Raman,[7,25,31] NMR,[32−34] magnetic susceptibility,[5,25,35−37] MCD,[38] ENDOR/ESEEM,[39] and EXAFS.[40−43] From these studies and from the X-ray structures of KBPAP and its phosphate and tungstate complexes,[12,13] the structure of the dinuclear metal center in PAP is now well characterized. Relatively little is known, however, about the mechanism by which PAPs catalyze the hydrolysis of phosphate esters. From a chiral substrate experiment with BSPAP, it is known that hydrolysis results in net inversion of the configuration around phosphorus, which strongly suggests the direct attack of water/hydroxide on the phosphate ester without the formation of a phosphorylated enzyme intermediate.[44] Stopped-flow measurements on the reaction of phosphate with uteroferrin suggest a rapid binding of phosphate to the Fe^{2+}.[45,46] A mechanism has been proposed in which the phosphate ester also rapidly binds to the Fe^{2+}, followed by attack of an Fe^{3+}-bound hydroxide ion.[13,44,47] The

enzyme is thus postulated to make use of the strong Lewis acidity of Fe^{3+} to generate a hydroxide ion, even at the weakly acidic pH where it is active (pH optimum ∼6).

In general, trivalent metal ions are stronger Lewis acids than divalent metal ions. As such, they are widely used as catalysts in organic synthesis, e.g., the Friedel−Crafts catalyst $AlCl_3$.[48] Ligand exchange reactions are, however, generally slower at trivalent metal ions than at divalent metal ions.[49] This fact has been put forward to explain why the vast majority of metallo-hydrolases contain divalent metal ions, in particular zinc and magnesium.[50] Despite this apparent disadvantage of trivalent metal ions, several enzymes are now known to make use of the strong Lewis acidity of a trivalent metal ion while exhibiting fairly high turnover numbers: the PAPs and possibly other phosphatases with the same sequence motif (Fe^{3+}),[2,22] nitrile hydratases (Fe^{3+} or Co^{3+}),[51,52] and intradiol dioxygenases (Fe^{3+}).[53,54]

A classical approach for investigating the role of a metal ion in enzymatic catalysis is substitution by another metal and characterization of the resulting perturbation in spectroscopic and/or enzymatic properties. We have recently reported that both of the iron ions in BSPAP can be specifically replaced by other metal ions.[8] By preparing GaFe-BSPAP and GaZn-BSPAP, we showed that the ferric iron can be replaced by Ga^{3+} without major effects on kinetics parameters and pH optima. The coordination chemistry of Fe^{3+} and Ga^{3+} is, however, known to be very similar. Ga^{3+} and Fe^{3+} have similar ionic radii, they show similar ligand exchange rates, and their Lewis acidities are comparable.[55] In the present study, we probe the role of the trivalent metal ion by the preparation and characterization of metal-substituted BSPAP forms of the general formula M^{III}-Zn^{II}, with two trivalent metal ions that differ significantly from iron: aluminum and indium. The ionic radius of Al^{3+} is smaller than that of Fe^{3+}, while that of In^{3+} is larger.[56] $[Al(H_2O)_6]^{3+}$ is also significantly less acidic than is $[Fe(H_2O)_6]^{3+}$ or $[Ga(H_2O)_6]^{3+}$.[57] When studying catalytic reactions involving ligand exchange at a metal, it is important to consider the ligand exchange rates typical of that metal ion. Thus, ligand exchange rates are typically slow for Al^{3+} complexes in comparison to the corresponding Fe^{3+} or Ga^{3+} complexes, while they are higher for In^{3+} complexes.[49,58−60] As a result, our findings, that the $Al^{III}Zn^{II}$-BSPAP form is ∼60% active as the $Fe^{III}Zn^{II}$ and $Ga^{III}Zn^{II}$ forms, with a k_{cat} of ∼2000 s^{-1}, while the $In^{III}Zn^{II}$ form is totally inactive, are rather surprising. This first demonstration of an *active* aluminum-containing enzyme is also in conflict with the axiom that the relative slow ligand exchange

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M^{III}*Zn*^{II} *Purple Acid Phosphatase*

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rates of aluminum render this metal ion useless in the active sites of enzymes.[50,55] Our findings suggest that the rate-limiting step in the catalytic mechanism of BSPAP may *not* be a ligand substitution reaction at the trivalent metal ion. Possible roles for the trivalent metal ion in phosphate ester hydrolysis are discussed.

Experimental Section

General. Unless stated otherwise, protein solutions contained 40 mM sodium acetate, 1.6 M KCl, and 20% (v/v) glycerol, pH 5.0. Optical spectra were measured on an HP8452A diode array spectrophotometer. Bovine spleen purple acid phosphatase was isolated as previously described.[8,61] Preparations had $A_{280 \, nm}/A_{536 \, nm}$ ratios of ∼14. Apo-BSPAP, FeZn-BSPAP, and GaZn-BSPAP were prepared as previously described.[8] Apo-BSPAP typically contained 0.05 mol of Fe per mole of protein and had a residual phosphatase activity of ∼0.1%. Protein concentrations were determined by measuring the absorption of the tyrosinate-to-Fe^{3+} charge-transfer band at 536 nm (ϵ = 4080 M^{-1} cm^{-1}) for FeFe-BSPAP and FeZn-BSPAP and the absorbance at 280 nm (ϵ = 60 000 M^{-1} cm^{-1}) for the apo-enzyme and the other metal-substituted forms of BSPAP. For all experiments involving apo-BSPAP, plastic disposable labware was boiled in 5% HCl and rinsed with Millipore water, while glassware was stored in 10% HCl and rinsed with Millipore water just before use. Sephadex G-25 columns (Pharmacia) were washed with 2−3 volumes of 2 mM 1,10-phenanthroline, followed by several volumes of Millipore water and buffer. Buffers were treated with Chelex-100 (BioRad) to remove metal impurities.

Enzyme Kinetics. Activity assays, determination of kinetics parameters K_M and k_{cat}, and determination of pH profiles were done as previously described using *p*-nitrophenyl phosphate (*p*-NPP) as substrate at pH 6.0.[8] Fluoride inhibition was studied in 100 mM NaOAc, 200 mM KCl, pH 5.0, by measuring the formation of phenol from the hydrolysis of phenyl phosphate at 278 nm (ϵ = 870 M^{-1} cm^{-1}) for nine substrate concentrations and four or five different fluoride concentrations. The entire data set was fit to the Michaelis−Menten equation for mixed-competitive inhibition using the program MacCurveFit 1.4 (Kevin Raner Software).

Time Dependence of the Formation of M^{III}Zn^{II}-BSPAP. A 4.2 μM apo-BSPAP solution was incubated with 200 μM ZnCl$_2$ and 200 μM of the trivalent metal (GaCl$_3$, FeCl$_3$, Al(NO$_3$)$_3$, InCl$_3$, or no trivalent metal) at 33 °C. At several times after metal addition, an aliquot was taken and assayed for activity using the continuous assay in a buffer containing 100 mM Na-MES, 200 mM KCl, and 10 mM *p*-NPP, pH 6.00, at 22 °C. An effective $\epsilon_{410 \, nm}$ of 1266 M^{-1} cm^{-1} was used for *p*-nitrophenol at this pH.

Titration of Indium Binding to Apo-BSPAP. Apo-BSPAP (12.8 μM) was incubated with 98 μM ZnCl$_2$ and various amounts of InCl$_3$ for 5.5 h at 22 °C. Then, 95 μM GaCl$_3$ was added. After incubation of the solution for at least 20 min at 22 °C, the activity of the solution was measured using the continuous assay in a buffer containing 100 mM Na-MES, 200 mM KCl, and 10 mM *p*-NPP, pH 6.00, at 22 °C.

Preparation of AlZn-BSPAP. Al(NO$_3$)$_3$ (∼200 μM) and ZnCl$_2$ (∼200 μM) were added to a solution of apo-BSPAP (∼30 μM) and incubated at 37 °C for 8 h. Precipitated protein was removed by centrifugation (15000*g*). Excess metal ions were removed by repeated concentration/dilution with metal-free buffer using a Centricon-30 concentrator (Amicon).

Preparation of InZn-BSPAP. InCl$_3$ (∼200 μM) and ZnCl$_2$ (∼200 μM) were added to a solution of apo-BSPAP (∼40 μM) and incubated at 22 °C for 3 h. Precipitated protein was removed by centrifugation (15000*g*). Excess metal ions were removed by repeated concentration/dilution with metal-free buffer using a Centricon-30 concentrator.

Metal Analyses. Metal analyses for Fe, Al, and Zn were performed on a Hitachi180-80 polarized Zeeman atomic absorption spectrometer equipped with a graphite furnace. Adventitious metal ions were removed from buffer by passage through a Chelex-100 column. Analysis of indium was performed by instrumental neutron activation analysis
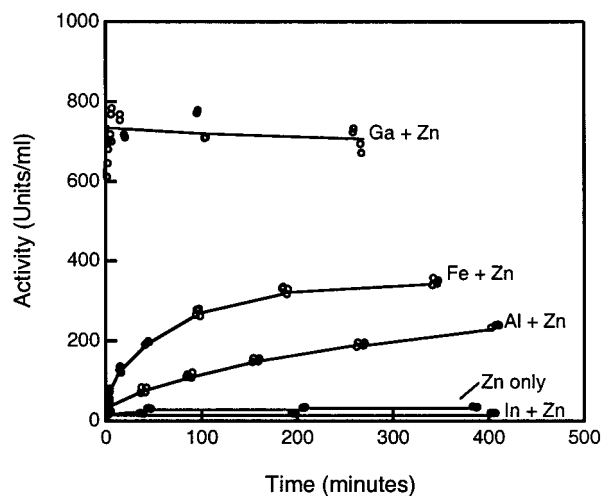
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Figure 1. Time dependence of the formation of phosphatase activity after incubation of apo-BSPAP (4.2 μM) with 200 μM ZnCl$_2$ and 200 μM GaCl$_3$, 202 μM FeCl$_3$, 202 μM Al(NO$_3$)$_3$, 201 μM InCl$_3$, and no trivalent metal ion added. Incubations were performed at 33 °C in a solution containing 35 mM sodium acetate, 1.4 M KCl, and 17% (v/v) glycerol, pH 5.0. Activity assays were performed at 22 °C in 100 mM Na-MES, 200 mM KCl, pH 6.00, with 10 mM *p*-NPP.

(INAA) at the Interfacultair Reactor Instituut, Delft, The Netherlands. Samples for INAA were desalted by repeated concentration/dilution with Millipore water using a Centricon-30 concentrator and lyophilized by freeze-drying in polyethylene INAA vials.

Results

In a previous study, it was shown that Fe^{III}Zn^{II}-BSPAP and Ga^{III}Zn^{II}-BSPAP can be obtained simply by the simultaneous addition of the trivalent metal and Zn^{2+} to apo-BSPAP, followed by removal of the excess metal ions.[8] These results showed that the apo-enzyme contains two clearly different metal sites: a "ferric" site with a high affinity for trivalent metals and a "ferrous" site with a high affinity for divalent metals. With the goal of resolving the role of the ferric ion in phosphate ester hydrolysis, it was decided to prepare a series of metal-substituted BSPAP forms of the general formula M^{III}Zn^{II}-BSPAP. Zn^{2+} was chosen as the invariant divalent metal instead of the native Fe^{2+}, since metal-substituted forms having Fe^{2+} at the divalent metal site may, in principle, disproportionate, resulting in the reformation of some of the native Fe^{III}Fe^{II}-BSPAP form. Such a disproportionation has been observed for GaFe-BSPAP.[8]

Apo-BSPAP was incubated with a ∼40-fold excess of ZnCl$_2$ and a ∼40-fold excess of either FeCl$_3$, GaCl$_3$, Al(NO$_3$)$_3$, InCl$_3$, or no trivalent metal at 33 °C, and the formation of phosphatase activity was monitored as a function of time after metal addition (Figure 1). Under these conditions, the addition of gallium results in an almost immediate formation of GaZn-BSPAP activity (within 3 min). The addition of Fe^{3+} also results in the formation of phosphatase activity, but the formation of FeZn-BSPAP is much slower. Surprisingly, the addition of Al^{3+} also resulted in phosphatase activity. The formation of this putative AlZn-BSPAP form is even slower than that of FeZn-BSPAP. The addition of either In^{3+} and Zn^{2+}, or Zn^{2+} in the absence of any trivalent metal, gave rise to a low level of phosphatase activity (∼4% of GaZn-BSPAP activity), which did not increase further with time. Furthermore, this phosphatase activity disappeared almost completely after incubation for 10 min in the presence of the strong reductant sodium dithionite, which strongly suggests that it is due to FeZn-BSPAP. This is consistent with the amount of residual iron that was determined

Table 1. Kinetics Parameters for Various Metal-Substituted Forms of Bovine Spleen Purple Acid Phosphatase[a]

enzyme	$k_{cat} \times 10^{-3}$ (s^{-1})	K_M (mM)	K_i (F$^-$) (μM)[e]
Al^{III}Zn^{II}	1.90 (0.04)[b]	4.2 (0.1)[b]	3 (0.2)
Ga^{III}Zn^{II}	3.09 (0.44)[b,d]	5.5 (0.4)[b,d]	140 (70)
In^{III}Zn^{II}	<0.02[b]	nd	nd
Fe^{III}Zn^{II}	2.84 (0.18)[b,d]	3.3 (0.4)[b,d]	200 (30)
Fe^{III}Fe^{II} (native)	1.76 (0.09)[c,d]	1.2 (0.2)[c,d]	nd

[a] Numbers in parentheses are standard deviation values. [b] Assays were performed at 22 °C and pH 6.00 in a buffer containing 100 mM Na-MES and 200 mM KCl with *p*-NPP as substrate. [c] Assays were performed at 22 °C and pH 6.0 in a buffer containing 100 mM Na-MES, 200 mM KCl, 15 mM sodium ascorbate, and 0.2 or 2 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ with *p*-NPP as substrate. [d] Data from ref 8. [e] Assays were performed at 22 °C and pH 5.0 in a buffer containing 100 mM NaOAc and 200 mM KCl with phenyl phosphate as substrate.

for this apo-BSPAP preparation, ~0.05 Fe per protein. Thus, the addition of either Zn^{2+} alone or In^{3+} in combination with Zn^{2+} does not result in any phosphatase activity other than that attributable to a small impurity of FeZn-BSPAP. It should be noted that apo-BSPAP is not fully stable under the conditions used in this experiment (33 °C). On a time scale of hours, substantial denaturation of apo-BSPAP occurs, so that denaturation of apo-protein competes with formation of FeZn-BSPAP and AlZn-BSPAP. The final activity levels observed in this experiment (Figure 1), therefore, do not reflect the relative specific activities of the purified GaZn-BSPAP, FeZn-BSPAP, and AlZn-BSPAP derivatives.

AlZn-BSPAP. The slow formation of AlZn-BSPAP requires either very long incubation times or the use of higher temperatures. AlZn-BSPAP was prepared by the incubation of apo-BSPAP with 200 μM Al(NO$_3$)$_3$ and 200 μM ZnCl$_2$ at 37 °C. When no further increase of phosphatase activity was measured (after ~8 h), denatured protein was removed by centrifugation, and the supernatant was concentrated/diluted several times with metal-free buffer to remove excess aluminum and zinc. Metal analysis showed the presence of 1.0 mol of aluminum, 0.7 mol of zinc, and 0.1 mol of iron per mole of protein. Like GaFe-BSPAP and GaZn-BSPAP[8], AlZn-BSPAP lacks the characteristic purple color of the FeFe- and FeZn-BSPAP forms and is colorless (Figure S1, Supporting Information), indicating the absence of the Fe(III) at the trivalent metal site. The very weak residual band at ~550 nm is probably due to a small amount (~10%) of FeZn-BSPAP present in this preparation. This iron probably also gives rise to the small EPR signal at $g = 4.3$ which is present in the EPR spectrum of AlZn-BSPAP. More importantly, the typical EPR spectrum of the native, antiferromagnetically coupled Fe^{III}Fe^{II} cluster with g-values of 1.87, 1.74, and 1.58 is completely absent in the spectrum of AlZn-BSPAP (Figure S2, Supporting Information). The specific activity of AlZn-BSPAP is comparable to that of the native FeFe-BSPAP and ~60% that of FeZn-BSPAP. The addition of dithionite to FeFe- and FeZn-BSPAP has been shown to result in the reduction of the ferric iron and inactivation of these BSPAP forms[8], but AlZn-BSPAP retained ~90% of its phosphatase activity in the presence of dithionite, which is consistent with the replacement of Fe^{3+} by Al^{3+}. The 10% decrease of phosphatase activity in the presence of dithionite is consistent with the residual 0.1 Fe detected by AAS and the residual features observed in the optical and EPR spectra. Like those of FeZn- and GaZn-BSPAP[8], the phosphatase activity of AlZn-BSPAP was insensitive to oxidation by hydrogen peroxide, indicating that the native Fe^{2+} has been replaced by Zn^{2+}.

Table 1 compares the kinetics parameters of the various M^{III}-Zn^{II} forms and the native Fe^{III}Fe^{II} enzyme for the hydrolysis of
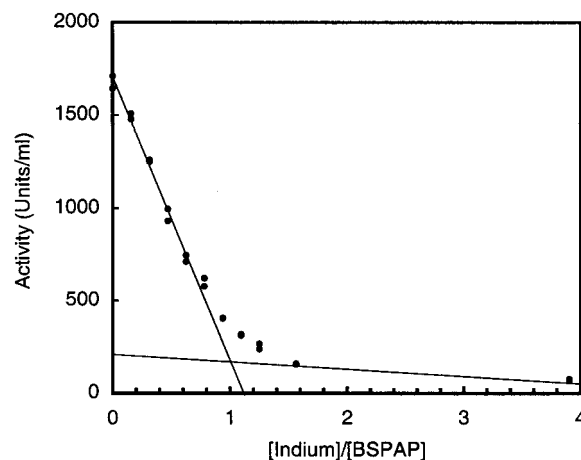

Figure 2. Titration of indium binding to apo-BSPAP. Apo-BSPAP (12.8 μM) was incubated with 98 μM ZnCl$_2$ and various amounts of InCl$_3$ at 22 °C for 5.5 h in a solution containing 35 mM sodium acetate, 1.4 M KCl, and 17% (v/v) glycerol, pH 5.0. Next, 95 μM GaCl$_3$ was added. Phosphatase activity was determined ~20 min after gallium addition. Activity assays were performed at 22 °C in 100 mM Na-MES, 200 mM KCl, pH 6.00, with 10 mM *p*-NPP.

p-NPP at 22 °C and pH 6.00. Overall, the kinetics parameters of AlZn-BSPAP are remarkably similar to those of GaZn-BSPAP and FeZn-BSPAP. The K_M value is close to that of FeZn-BSPAP, while k_{cat} is decreased 1.5-fold compared to those of FeZn-BSPAP and GaZn-BSPAP. It should be noted, however, that the aluminum-substituted enzyme is still a very efficient phosphatase, having a turnover rate of nearly 2000 s^{-1}. Fluoride, which is an uncompetitive inhibitor at pH 5.0,[62] binds much more strongly to AlZn-BSPAP than to GaZn-BSPAP or FeZn-BSPAP. The pH dependence of the phosphatase activity of AlZn-BSPAP was determined for the hydrolysis of *p*-NPP at a constant *p*-NPP concentration of 50 mM (not shown). The pH profile is bell-shaped with apparent pK_a values of 5.2 and 7.1, and is thus very similar to that of FeZn-BSPAP under identical conditions (pK_as of 5.3 and 7.1).[8]

InZn-BSPAP. The absence of any activity after incubation of apo-BSPAP with indium and zinc means either that apo-BSPAP is not able to bind indium or that In^{III}Zn^{II} BSPAP is formed but is inactive. If In^{3+} is able to bind at the trivalent metal site, it should prevent the binding of gallium and, therefore, the development of phosphatase activity resulting from GaZn-BSPAP. Figure 2 shows a titration experiment in which 12.8 μM apo-BSPAP was incubated with excess Zn^{2+} and various amounts of In^{3+} for 5.5 h at 22 °C. Subsequently, 95 μM Ga^{3+} was added, and the phosphatase activity was determined after another 10−20 min incubation time. Figure 2 shows that the binding of one In^{3+} ion per apo-BSPAP precluded the formation of active GaZn-BSPAP. Independent evidence for the formation of BSPAP with a dinuclear In^{III}Zn^{II} center came from metal analysis of InZn-BSPAP that had been prepared by the addition of excess In^{3+} and Zn^{2+}. Metal analysis showed that, even after repeated concentration/dilution in metal-free buffer, the BSPAP samples still contained equimolar amounts of indium (1.2 mol/mol of protein) and zinc (1.1 mol/mol of protein) and only very little iron (0.05 mol/mol of protein).

Discussion

Preparation and Characterization of AlZn-BSPAP and InZn-BSPAP. In this study we demonstrate that the "ferric"

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Table 2. Comparison of the Catalytic Activity of $M^{III}Zn^{II}$-BSPAP and Some Properties of the Trivalent Metal

M(III)	k_{cat} (s^{-1})	K_M (mM)	ionic radius (Å)^a	pK_a [M(H$_2$O)$_6$]$^{3+}$ ^{b}	$k_{exchange}$ (s^{-1})		
					[M(H$_2$O)$_6$]$^{3+}$	[M(H$_2$O)$_5$OH]$^{2+}$	M(acac)$_3$^{c}
Al	1.9×10^3	4.2	0.54	5.5	1.3^c		9.1×10^{-5}
Fe	2.8×10^3	3.3	0.65	2.7	1.6×10^2 ^{c}	1.2×10^5 ^{e}	3.3×10^{-3}
Ga	3.1×10^3	5.5	0.62	3.0	4.0×10^2 ^{c}	$(0.6-2.0) \times 10^5$ ^{f}	1.6×10^{-3}
In	<20	nd	0.80	4.4	$\geq 10^7$ ^{d}		2

^{a} From ref 56. ^{b} All values converted from ref 57 to 0.16 M ionic strength. ^{c} From ref 60 and references therein. ^{d} From ref 59. ^{e} From ref 49. ^{f} From ref 72.

metal site of BSPAP can accommodate a variety of trivalent metal ions. Gallium, iron, and aluminum all yield active enzymes. Indium does bind to apo-BSPAP, but In^{III}Zn^{II} BSPAP is inactive. The only proteins for which the interaction with trivalent metal ions have been studied extensively are the Fe^{3+} transport/binding proteins transferrin, lactoferrin, and ovotransferrin.[63] In these proteins, the trivalent metal is coordinated by two tyrosinates, one histidine, one carboxylate, and a nonprotein ligand, carbonate,[64,65] while the Fe^{3+} in the purple acid phosphatases is coordinated by one tyrosinate, one histidine, one terminal carboxylate, one bridging carboxylate, probably a bridging hydroxide, and either water or hydroxide.[12,13] Aluminum, gallium, and indium all bind quite strongly to transferrin, and a free energy relationship has been observed between the stability constants for hydroxide and phenolate binding and their binding constants to transferrin, suggesting that electrostatic interactions rather than, e.g., ionic radii determine the binding strength.[66] The rate of formation of the M^{III}Zn^{II} BSPAP strongly depends on the trivalent metal, with rates decreasing in the order Ga^{3+} ≫ Fe^{3+} > Al^{3+}. The rapid formation of GaZn-BSPAP was used to advantage in studying the formation of InZn-BSPAP, because it allowed the amount of free trivalent metal sites to be determined by their reaction with gallium to form the active GaZn-BSPAP species. For transferrin, the relative order of the rates of complex formation differs from that found for apo-BSPAP (Ga > Al > Fe > In).[67] For both BSPAP and transferrin, these rates of complex formation do not follow the trends found for ligand exchange rates for trivalent metal ions, indicating that some process other than a simple ligand exchange is determining these rates.

Our previous work on the gallium-substituted BSPAP forms showed that the kinetics parameters and the pH profiles of FeFe-, GaFe-, FeZn-, and GaZn-BSPAP are similar but not identical.[8] The small differences that were detected were consistent. Thus, the same effects on k_{cat} and K_M and the pH profiles were observed for the Fe^{3+}-to-Ga^{3+} substitution when going from FeFe-BSPAP to GaFe-BSPAP and when going from FeZn-BSPAP to GaZn-BSPAP. The same is true for the Fe^{2+}-to-Zn^{2+} substitution. The differences between the various M^{III}Zn^{II} BSPAP forms may, therefore, be ascribed with some confidence to chemical differences that are due to the identity of the trivalent metal, and not to some artifact introduced by their preparation. The value of k_{cat} is calculated on the basis of the amount of protein. Since our AlZn-BSPAP preparations contain 0.7 Zn

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per protein, it may be argued that one should calculate k_{cat} on the basis of the amount of zinc. Doing this would increase the value of k_{cat} to 2700 s^{-1}, which would mean that the activity of AlZn-BSPAP is the same as those of FeZn-BSPAP and GaZn-BSPAP.

Relating Enzymatic Properties to General Properties of the Trivalent Metal. The trivalent metals used in this study are all spherically symmetrical, having either fully filled (d^{10}: Al^{3+}, Ga^{3+}, and In^{3+}) or half-filled, high-spin d-shells (d^5: Fe^{3+}). The absence of specific ligand field effects facilitates the comparison of the enzymatic properties of these metal-substituted enzymes to the general properties of the trivalent metal. In Table 2, some of these properties (ionic radius in octahedral complexes, ligand exchange rates for [M(H$_2$O)$_6$]$^{3+}$, [M(H$_2$O)$_5$OH]$^{2+}$, and [M(acac)$_3$], and pK_a values for [M(H$_2$O)$_6$]$^{3+}$) are compared with the enzymatic properties k_{cat} and K_M for the M^{III}Zn^{II} BSPAP form containing the same trivalent metal.

Stability constants for hydroxide and other oxygen and nitrogen ligands are similar for iron(III) and gallium(III) but lower for the corresponding aluminum complexes.[58] The greater acidity of [Fe(H$_2$O)$_6$]$^{3+}$ relative to that of [Al(H$_2$O)$_6$]$^{3+}$ has been correlated with the greater electronegativity of Fe(III) ($\chi_{Fe(III)}$ = 1.96; $\chi_{Al(III)}$ = 1.61), which means that the Fe–O bond has some covalent character, while the Al–O bond is purely electrostatic. Whether p-NPP binds to the trivalent metal ion remains to be established, but the very similar substrate affinities found for AlZn-, FeZn-, and GaZn-BSPAP suggest that such an M^{III}–O bond does not contribute significantly to substrate binding. The finding that AlZn-BSPAP is inhibited much more strongly by fluoride than GaZn- and FeZn-BSPAP indicates that the noncompetitive inhibition is due to fluoride binding at the trivalent metal ion, where it is likely to replace a coordinated water/hydroxide. The stronger binding of fluoride to aluminum is in agreement with the hard–soft acid–base theory: fluoride is a hard base and Al^{3+} is a harder acid than both Fe^{3+} and Ga^{3+}.

The acidic limb of the pH optimum of BSPAP has been attributed to a M(III)-coordinated water ligand with a pK_a around 5 in the enzyme–substrate complex[47]. The similar pH profiles for FeZn-BSPAP and AlZn-BSPAP seem to be in conflict with this proposal, since the pK_a of the M(III)-coordinated water would be expected to be higher for AlZn-BSPAP than for FeZn-BSPAP. The pH profile may, therefore, be the result of the (de)-protonation of amino acid side-chain groups or substrate. As will be discussed below for ligand exchange rates, some care should be taken when comparing the thermodynamic properties of, e.g., the hexaaqua complexes with those of the metal–protein complexes. It may be that, in complexes with several anionic ligands, these trends in pK_a values differ from the trend in the pK_a values of the first deprotonation step in the hexaaqua complexes, as shown in Table 2.

In most discussions of the relationship between ligand exchange rates and the nature of the metal ion, the water

exchange rates of the hexaaqua complexes of the metal ions are compared.[50,55,68] The water exchange rates for the trivalent metal ions studied in this work decrease in the order $In^{3+} \gg Ga^{3+}, Fe^{3+} > Al^{3+}$, which also reflects the increase in charge/ionic radius ratio for these metal ions.[58] The difference between the k_{cat} for AlZn-BSPAP (1900 s^{-1}) and the water exchange rate for $Al(H_2O)_6$ ($\sim$1 s^{-1}) is remarkably large. At first glance, these facts seem incompatible with a catalytic mechanism that includes a ligand exchange reaction at the trivalent metal. It is dangerous, however, to compare ligand exchange rates for two complexes with the same metal ion but with a different ligand environments. In purple acid phosphatases, the ligand will not experience the full 3+ charge of the trivalent metal as in the hexaaqua complexes, because the trivalent metal is coordinated by several negatively charged ligands: one tyrosinate, one end-on bound carboxylate, a bridging carboxylate, and probably also a bridging hydroxide. The effect of such a negatively charged ligand can be clearly seen by comparing the water exchange rates for $[M(H_2O)_6]^{3+}$ and the corresponding $[M(H_2O)_5OH]^{2+}$ complexes (Table 2). It has also been reported that Fe^{III}-EDTA and Fe^{III}-porphyrins have much faster water exchange rates of $\sim$10^5 s^{-1}.[69] On the other hand, in the all mechanisms proposed thus far for the PAPs (vide infra), a negatively charged phosphate rather than a neutral water ligand needs to be displaced by a water molecule, which might be expected to slow the exchange reaction.[13,42]

Since so many factors may contribute to the rate of ligand exchange, the absolute rates of ligand exchange reactions in model complexes cannot be directly correlated with k_{cat} values. If one compares ligand exchange rates for various trivalent metals with the same ligand environment, however, the relative rates between corresponding complexes of the various metals seem fairly constant.[60] If a ligand exchange reaction at the trivalent metal ion is the rate-determining step in PAP catalysis, k_{cat} would be predicted to be similar for FeZn-BSPAP and GaZn-BSPAP but at least a factor of 10 lower for AlZn-BSPAP. The relatively high activity of AlZn-BSPAP is, therefore, inconsistent with a rate-limiting ligand exchange reaction at the trivalent metal ion, although it cannot be excluded that ligand exchange is partially rate-limiting for the aluminum-containing enzyme.

The InZn-BSPAP is the first example of a purple acid phosphatase for which substitution of the ferric iron by another trivalent metal results in inactivation of the enzyme. In^{3+} is a weaker Lewis acid then Ga^{3+}, Fe^{3+}, and Al^{3+}, which may be the reason for its inactivity. Since the ionic radius of In^{3+} is also larger, we cannot exclude the possibility that the binding of In^{3+} causes structural changes in the active site that affect the catalytic activity.

Figure 3 shows three different mechanistic proposals for the role of the two metal ions in hydrolysis. All mechanisms have as a common feature the initial coordination of the phosphate ester to the divalent metal ion. In mechanism 1, proposed by Witzel and co-workers,[13,47] the phosphate ester is subsequently attacked by the hydroxide, which is bound to the ferric iron in a monodentate fashion. The final step is then release of phosphate. Mechanism 2 has been proposed for protein phosphatase 1 and calcineurin, on the basis of the X-ray structures of their oxoanion complexes,[18,19] and recently also for uteroferrin.[42] In this mechanism, the substrate binds in a bridging mode to both metal ions. The μ-OH bridge is then activated to act as the nucleophile that attacks the phosphate ester. Both mecha-
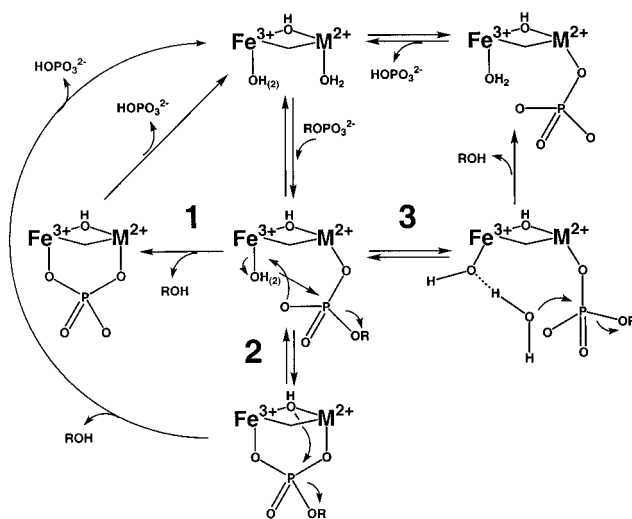

Figure 3. Three mechanistic proposals for the role of the PAP metal center in the hydrolysis of phosphate monoesters. Starting at the top center of the figure with the free enzyme, all mechanisms have the initial coordination of the phosphate ester to the divalent metal ion in common. In mechanism 1, the phosphate ester is subsequently attacked by the hydroxide that is coordinated monodentately to the ferric iron. In mechanism 2, the phosphate ester binds in a bridging mode to both metal ions and is then attacked by the bridging hydroxide. In mechanism 3, an Fe^{3+}-coordinated hydroxide acts as a general base to deprotonate a water in the second coordination sphere of the metal, which then attacks the phosphate ester coordinated to the divalent metal ion.

nisms 1 and 2 involve ligand substitution reactions at the trivalent metal ion in which phosphate is replaced by a water/hydroxide ligand. The similar k_{cat} values for AlZn-, FeZn-, and GaZn-BSPAP suggest that this release of phosphate is not the rate-determining step in catalysis, which leaves the hydrolysis reaction itself as the most likely rate-limiting step for both mechanisms 1 and 2.

A third mechanistic possibility is that ligand substitution at the trivalent metal ion does not occur during catalysis (mechanism 3). In this mechanism, the hydroxide that is coordinated to the trivalent metal (either end-on or bridging the two metals) acts as a general base to deprotonate a second water molecule, which then attacks the phosphate ester. In the final step, phosphate is released from the divalent metal ion. A similar role for the trivalent metal ion has recently been proposed for the Fe(III) or Co(III) ions in the enzyme nitrile hydratase.[51] Two classes of nitrile hydratases exist, one containing a low-spin Fe(III) metal center and one containing a low-spin Co(III) metal center. Both classes share sequence homology, and the coordination environments of the trivalent metals are believed to be similar. Typical values for k_{cat} are 1000–2000 s^{-1} for both enzyme classes. Since Co(III) complexes are known to be kinetically inert, it was proposed that the chemistry is taking place in the second coordination sphere of the trivalent metal, in a way similar to that depicted in mechanism 3.

Trivalent Metals as Lewis Acids in Enzyme Catalysis. In general, trivalent metal ions are better Lewis acids then divalent metal ions, and consequently one might expect the former to be widely distributed in enzymes. In fact, however, only a few well-characterized examples exist: the purple acid phosphatases and possibly other phosphatases with the same sequence motif, nitrile hydratase, and intradiol dioxygenases. In all these enzymes, the trivalent metal ions in these enzymes are bound via several anionic ligands, which will decrease their Lewis acidity compared to that of their hexaaqua complexes but at the same time increase their ligand exchange rates. Two options,

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therefore, seem open to metallohydrolases: using a divalent metal ion such as zinc with high ligand exchange rates in a coordination sphere that makes the metal ion as acidic as possible (e.g., all neutral histidine ligands, as in carbonic anhydrase), or using a trivalent metal ion such as Fe(III), which is intrinsically a much better Lewis acid, in an anionic ligand environment, which will increase the rate of ligand substitution at the trivalent metal ion.

Aluminum has no known biological role, despite the fact that it is the most abundant metal on earth and a good Lewis acid. Its slow ligand exchange rates are generally considered to be an important reason for its absence in biology; for example, in a review on the bioinorganic chemistry of aluminum, it was concluded that, "The slow ligand exchange rate renders Al^{3+} useless as a metal ion at the active sites of enzymes." [55] In another recent review on aluminum in biological systems, Williams also suggested that, "The Al^{3+} ion is not active in kinases in all probability because of its movement with a restricted rate constant of seconds. Here lies the problem of high valent metal ions." [70] The present results, however, demonstrate that an aluminum enzyme can be prepared that is an efficient catalyst of phosphate ester hydrolysis. The absence of aluminum in biology should, therefore, be attributed to other factors, such as its problematic transport and storage or its interference with Mg^{2+} at Mg^{2+} binding sites for phosphate esters (DNA/RNA, ATP, etc.).[71]

Conclusion. The phosphatase activity of AlZn-BSPAP is almost as great as that of the FeZn- and GaZn-BSPAP forms (k_{cat} ~2000 vs ~3000 s^{-1}), while $In^{III}Zn^{II}$-BSPAP is inactive. This first demonstration of an active aluminum-containing enzyme is in conflict with the axiom that the relative slow ligand exchange rates of aluminum render this metal ion useless in the active sites of enzymes.[55,70] Our findings suggest that the rate-limiting step in the catalytic mechanism of BSPAP may *not* be a ligand substitution reaction at the trivalent metal ion.

Supporting Information Available: Optical spectra of FeFe-BSPAP and AlZn-BSPAP (Figure S1) and EPR spectra of FeFe-BSPAP, FeZn-BSPAP, and AlZn-BSPAP (Figure S2) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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